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DIFFERENTIAL RESPONSES OF PHOTOSYSTEM II ACTIVITY TO PHOTOOXIDATION IN RED AND GREEN TISSUES OF *AMARANTHUS TRICOLOR* LEAVES

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Abstract

In order to study the antioxidative potential of amaranthine and its relationships with photoprotection, changes of PS II activity of red and green tissue in the same edible amaranth leaf were compared under photooxidation treatment induced by MV (methyl viologen) or H_2O_2. In the first 90 min of MV treatment, PS II maintained stable activity; the chlorophyll-fluorescence parameters were close to those of the controls. However, with the extension of treatment time, during 90-300 min of continuous photooxidative stress, F_v/F_m, Y (II) and Y (NPQ) of leaf discs decreased significantly, while Y (NO) and F_o dramatically increased, which showed that the activity of PS II suffered irreversible photooxidative damage, suppressed or even completely inhibited. Compared with the control, under 50 mmol·L^{-1} of H_2O_2 stress for 360 min, Y(NO) and F_o increased, accompanied by Y(NPQ) and the F_v/F_m decreases, while Y(II), qP and ETR were slightly increased, reflecting that treatment with 50 mmol·L^{-1} H_2O_2 promoted distribution of light energy in photochemistry reaction. After photooxidative treatments, a lower F_o and Y (NO) but higher F_v/F_m, Y (II), qP and Y (NPQ) was found in the red leaf discs compared with the green ones. The decrease and the decline rate of the amaranthine content in the green and red leaf discs implied that the PS II of green leaf discs have a higher sensitivity to photooxidative damage than the red ones. It is suggested that the higher content of amaranthine may be used as a detoxification mechanism to counteract photooxidation in red leaf.

Introduction

The mechanisms of photooxidative damage and defense against photooxidative stress have always been significant issues. Many biotic and abiotic stresses can increase the generation of reactive oxygen species (ROS) in plants (Wang *et al*., 2013; Shamim *et al*., 2013; Kanwal *et al*., 2013). The increase of reactive oxygen species in cells can induce (1) the generation of photooxidation stress and damage of organelles, in particular the photosynthetic apparatus; and (2) the rapid elimination of ROS by efficient antioxidative systems and refined regulatory mechanisms during photosynthesis (Foyer *et al.*, 1994). Moreover, antioxidative enzymes and low molecular-weight organic antioxidants distribute ubiquitously in the leaf, where plant pigments have a close association with antioxidative activity. Among all frequently-found coloration pigments, both carotenoids and anthocyanins are ascertained to efficiently scavenge free radicals in plants (Gould *et al.*, 2002; Tanaka *et al.*, 2008). However, there is still limited information about the anti-photooxidative capacity of another important secondary metabolite, amaranthine, a homologue of anthocyanidins in higher plants (Cai *et al.*, 1998; Wang *et al.*, 2006).

Amaranthine is a class of water-soluble red-violet pigments, accumulated in red roots, stems and leaves of plants of *Amaranthus* (Amaranthaceae), belonging to Betacyanins of Betalain (Cai *et al.*, 2001; Stintzing & Carle, 2004). As a secondary metabolite of nitrogenous plants, amaranthine is a potential antioxidant because of its abundance of hydroxyl and imino groups (Cai *et al.*, 1998; Strack *et al.*, 2003). Methods for extracting amaranthine and its chemical property analysis have been investigated recently (Zhan *et al.*, 2003; Cai *et al.*, 2005; Gu *et al.*, 2008). However, the accumulation of amaranthine in Amaranthaceae plants and its related physiological function have been rarely reported. Our previous study revealed that red edible amaranth can decrease H_2O_2 accumulation and increase heat resistance by accumulating amaranthine quickly (Shao *et al.*, 2008). One red cultivar of amaranth exhibited a red-green leaf phenotype, the proportion of red part and green part in the leaf being 1:2 (Xiao *et al.*, 2000). Leaf discs of red or green parts were treated with H_2O_2 or MV solutions, which provides an efficient and in-depth approach to probe the antioxidative potential of amaranthine and the relationships with photoprotection. To compare antioxidative capability between red tissue and green tissue in the same leaf under photooxidation treatments, chlorophyll fluorescence measurements were carried out with an IMAGING-PAM chlorophyll fluorometer (Heinz Walz GmbH, Effeltrich, Germany). By using chlorophyll fluorescence technology in the present study, more experimental evidence was obtained to extend our understanding of the physiological functions of amaranthine, helping to elucidate the mechanisms that protect the photosynthetic apparatus in plants.

Materials and Methods

Plant materials and growth conditions: The variety of edible amaranth (*Amaranthus tricolor* L.) is a red cultivar of amaranth. Seeds of *Amaranthus tricolor* L. were obtained from Nanhai Dali Jiang Zhiqing Seed Company, Guangdong Province, China. Seeds were germinated in plastic potted with soil on the experimental field at South

China Normal University, Guangzhou, China. Whole plants of each cultivar were put into the growth chamber (*RXZ-500, Ningbo Jiangnan Instrument Factory*, Ningbo, China) for incubation at 30°C, with a 12-h photoperiod (about 100 μmol m^{-2} s^{-1} PPFD)。Leaf discs (12 mm diameter) of red part and green part were cut from the same leaf in 40-day-old plants.

Photooxidation treatment: Eight red or green leaf discs were soaked, adaxial side up, in a solution of 5 μmol·L^{-1} MV (1,10-dimethy-4.40-bipyridinium dichloride; Sigma Chemical Co., St Louis, MO, USA) or a solution of 50 mmol·L^{-1} H_2O_2. These solutions also contained 0.01% Triton-100. Leaf discs soaked in pure water (containing 0.01% Triton-100) were used as a control. The treatments were conducted in a growth chamber at a temperature of 30℃, light intensity of 30 μmol·m^{-2}·s^{-1} and a humidity of 60%. Chlorophyll fluorescence parameters of leaf discs were measured every 30 min for 5-6 times.

Chlorophyll fluorescence measurement: Fluorescence measurement was carried out with an IMAGING-PAM chlorophyll fluorometer (Heinz Walz GmbH, Effeltrich, Germany) as described by Siebke & Weis (1995) and Rascher *et al*., (2001). F_o (minimum fluorescence yield of a dark-adapted leaf) was measured with relatively weak measuring light pulses (<1 μmol·m^{-2}·s^{-1}) at a low frequency (1Hz). Maximal fluorescence yield of a dark-adapted leaf (F_m) was measured during an 800-ms exposure to a PPFD of approximately 2700 μmol·m^{-2}·s^{-1}. The intensity of continuous actinic illumination was adjusted to 185 μmol·m^{-2}·s^{-1}. All lighting (modulated measuring light, actinic light and saturation pulses for measurement of F_m and for the maximal fluorescence yield of a light-adapted leaf (F_m')) was provided from blue-light-emitting diodes (450 nm). All fluorescence measurements were started after 10-min dark adaptation. When performing a measurement, an area of interest (AOI) with a diameter of 1 cm was selected in the middle of the whole leaf disc. Values of the chlorophyll fluorescence parameters F_o, F_m and F_m', maximal PS II quantum yield (F_v/F_m), effective PS II quantum yield (FPSII), non-photochemical quenching (NPQ) and coefficient of photochemical quenching (qP) were the average of the AOI (n = 6). In addition, their images were simultaneously derived from the IMAGING-PAM software. The apparent absorption of the leaf surface is automatically calculated pixel by pixel from the red (R) and near infrared (NI) images using the formula: Abs = 1 - (R/NI). The definition of the apparent electron transport rate (ETR) assumes a uniform absorption of incident light over the whole sample area: ETR = Yield × PAR × 0.5 × Absorption, where Yield = $\triangle F/F_m$', PAR is the actinic light intensity, and 0.5 represents the 50% of the absorbed PAR distributed to PS II. Rapid light curve measurements (Schreiber *et al.*, 1997) were carried out using 20-s exposures to stepwise increased photon irradiance (0, 1, 21, 56, 111, 186, 281, 336, 461, 531, 611, 701, 801, 926, 1076 and 1251 μmol·m^{-2}·s^{-1}). Simultaneously, ETR values were obtained automatically using the Imaging-Win software.

Absorption spectra and contents of amaranthine: Amaranthine content was measured as described by Stintzing *et al*., (2002). Fresh leaf discs from the edible amaranth (0.2 g FW) were ground in a mortar and pestle. Amaranthine was extracted in 20ml methanol for 30 min followed by centrifugation for 10 min at 4°C and 12000 × *g*, and sampling of the supernatant. This process was repeated 3 times. Then the precipitation was soaked in 15ml distilled H_2O for 30 min. After centrifugation for 10min at 4°C and 12000 × g, the absorption spectra of supernatant were measured spectrophotometrically (450-700nm) with a Visible-UV spectrophotometer (UV-2550, Shimadzu, Japan) . The relative amount of amaranthine was indicated by OD_{538}.

Statistical analysis: Statistical analyses were conducted by using Origin 7.5 software (Microsoft Company, Northampton, MA, USA) and tested by a Duncan test.

Results

Absorption spectra and the content of amaranthine: The absorption spectra (450-700 nm) of methanol extracts from the red or green leaf discs of the same red cultivar of amaranth leaf were recorded (Fig. 1a). Red leaf discs showed a typical amaranthine absorption peak at 538 nm, whereas green leaf discs had no absorption peak in the same wavelength range. The relative amount of amaranthine in red leaf discs was significantly higher than that in green discs ($p<0.01$) (Fig. 1b).

Effects of photooxidative treatment on F_v/F_m: F_v/F_m represents the maximal PS II quantum yield. Fig. 2 shows an obvious decreasing trend in F_v/F_m in red and green discs during 300 min of MV or H_2O_2 treatment. In comparison with the control, after 120 min of treatment, F_v/F_m had a more significant decrease under MV treatment than under H_2O_2 treatment. After 300 min treatment, F_v/F_m in green tissue decreased by 84.4% (MV) and 49.8% (H_2O_2) in comparison with control, whereas F_v/F_m in red tissue only decreased by 67.1% (MV) and 38.1% (H_2O_2). Less decline of F_v/F_m in red leaf discs than in green leaf discs indicated that PS II in red discs was better protected against photooxidation.

Effects of photooxidative treatment on Y (II) and qP: Y (II) indicates the quantum yield of linear electron transport of PS II (Krall & Edward, 1992) and qP is a measure of the oxidation state of the primary quinine acceptor in PS II. During treatment with MV for 90 min, compared with control, Y (II) and qP showed a little increase in red leaf discs, though green leaf discs showed no significant difference (Fig. 3). Nevertheless, after continuous treatment from 120 to 300 min, Y (II) and qP decreased gradually, indicating that the reaction center of PS II and/or electron transport carriers might be damaged by MV treatment. The values of Y (II) and qP in green leaf discs were almost zero following 240 min treatment. In contrast, Y (II) and qP in red leaf discs remained at 0.084 and 0.201, respectively. However, treatment with 50 mmol·L^{-1} of H_2O_2 did not result in a distinct effect on Y (II) and qP in red and green leaf discs as MV treatment did. Y (II) and qP in the 2 types of leaf discs increased first and maintained a level slightly higher than that of the control in 360 min duration of treatment.

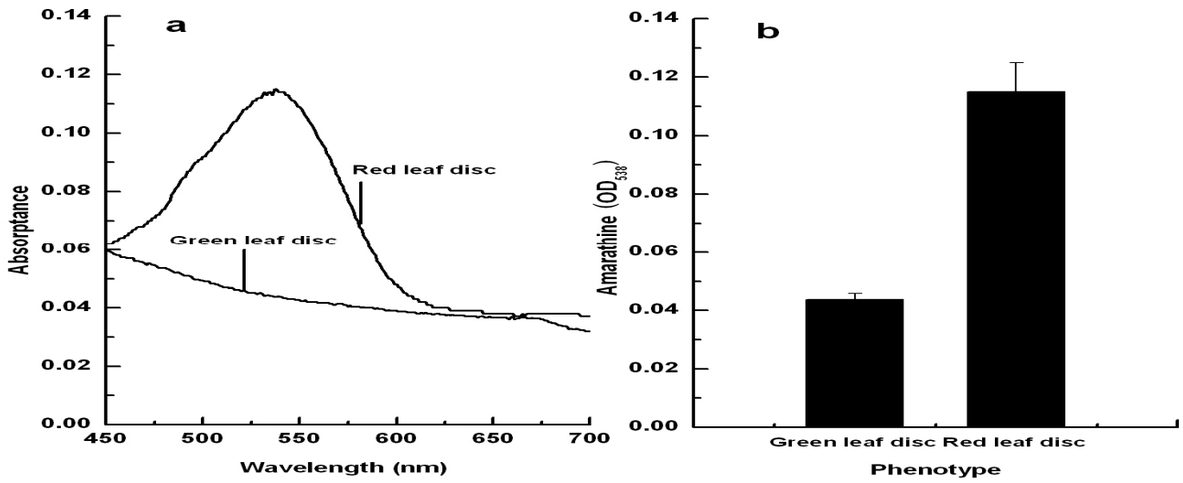

Fig.1. Absorption spectra and the contents of amaranthine in red or green discs sampled from the same leaf in a red cultivar of amaranth.

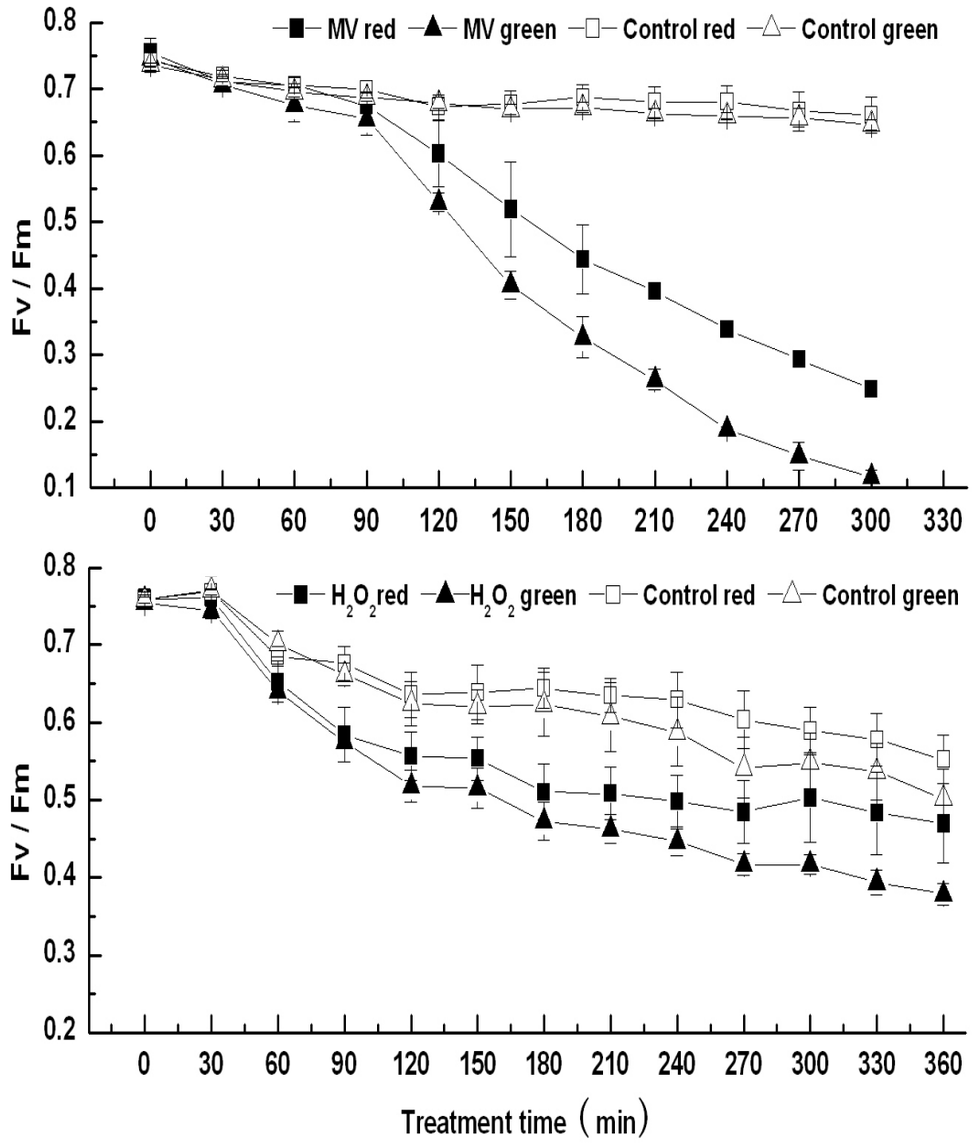

Fig.2. Effects of MV or H_2O_2 treatment on F_v/F_m in red or green discs from the same leaf in red cultivar of amaranth.

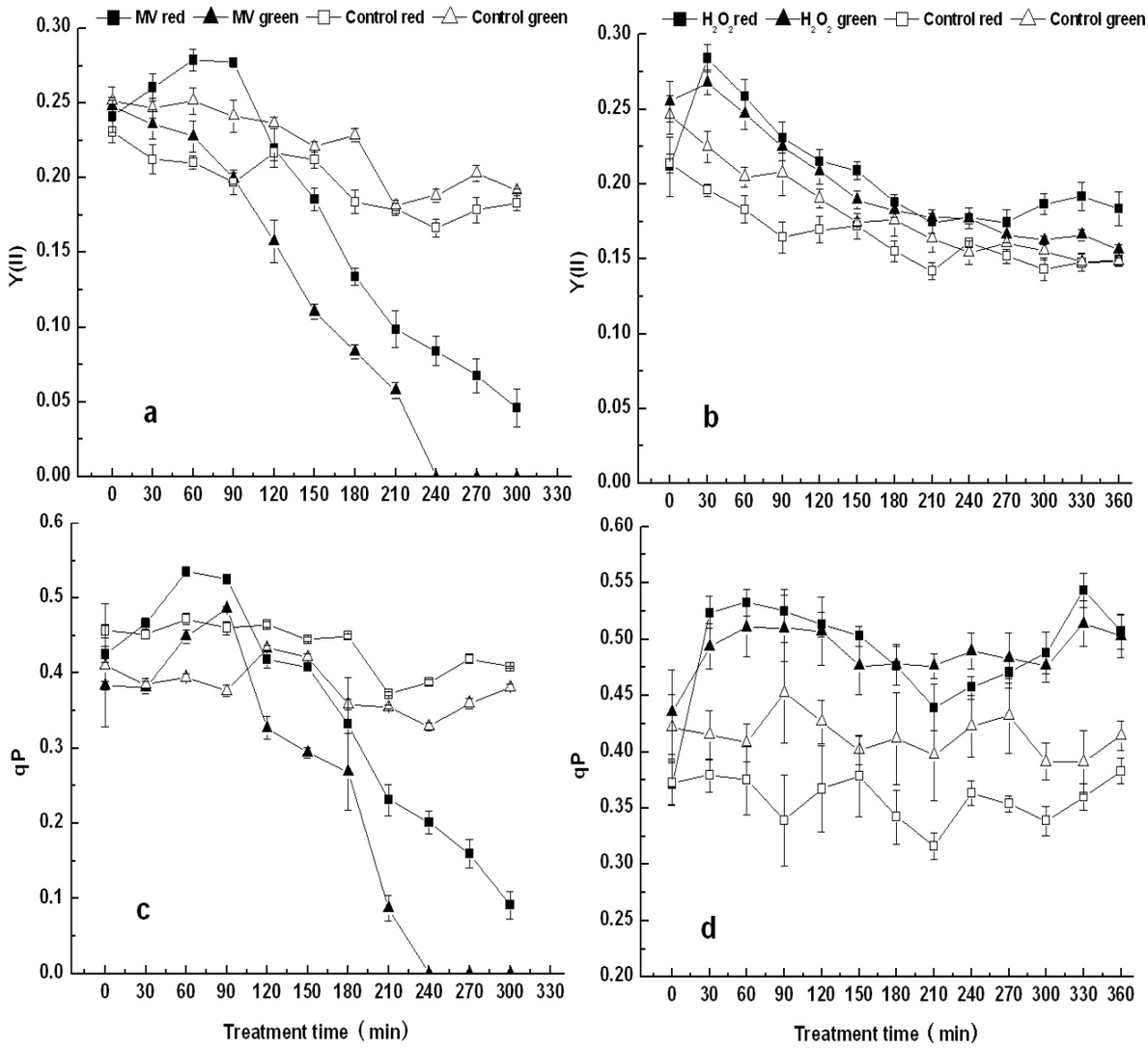

Fig. 3. Effects of MV or H_2O_2 treatment on Y (II) and qP in red or green discs from the same leaf in a red cultivar of amaranth.

Effects of photooxidative treatment on NPQ and Y (NO): Non-photochemical quenching (NPQ) means the potential of the plant to dissipate excess excitation energy via heat, and Y(NO) indicates damage because of excess light energy in PS II (Kramer *et al.*, 2004). During the process of photooxidative treatment of the two types of leaf discs (Fig. 4), the Y (NPQ) changes were opposite to those of Y (NO). The oxidative damage to the leaf discs by MV was significantly higher than that caused by H_2O_2 ($p<0.01$). In the initial MV treatment (0-90 min), Y (NPQ) of green and red leaf discs are the same as the control. At this time leaf discs may maintain PS II electron transport activity by adopting a higher photochemical quenching (qP) (Fig. 3). After 120 min treatment, Y (NPQ) had a sharp decline and Y (NO) rose markedly, suggesting that the antenna system had suffered serious oxidative damage.

Compared with the controls, after 120 min H_2O_2 treatment of the red and green leaf discs, Y (NPQ) decreased by 17.4% and 33.8%, Y (NO) increased by 19.1% and 28.5%, respectively, showing less damage in PS II than MV treatment. Compared with green leaf discs, in the subsequent 120-300 min of the MV and H_2O_2 treatments, red leaf discs had the higher Y (NPQ) and lower Y (NO) values, implying that the PS II of red leaf discs displayed better ability of resisting photooxidation than that of green discs.

Effects of photooxidation treatment on F_o: F_o is a relative measure of the original density of excitation energy in pigments in PS II (Georgieva & Yordanov, 1993). Damage of the PS II reaction center or reversible inactivation can cause an increase in F_o (Xu & Wu, 1996). In our experiment, the impact of H_2O_2 on F_o was not large, but it had a clear upward trend after 120 min of MV treatment (Fig. 5). The F_o of the green and red leaf discs increased by 30.3% and 15.1% compared with the control, respectively. The rise of F_o means that the PS II reaction centre had suffered oxidative damage. In the later MV treatment (120-300 min), F_o of the green leaf discs was always kept at a relatively high value, while that of the red discs only went up slowly. It suggested that thylakoid membranes of red leaf discs were more stable than in green leaf discs under oxidative stress.

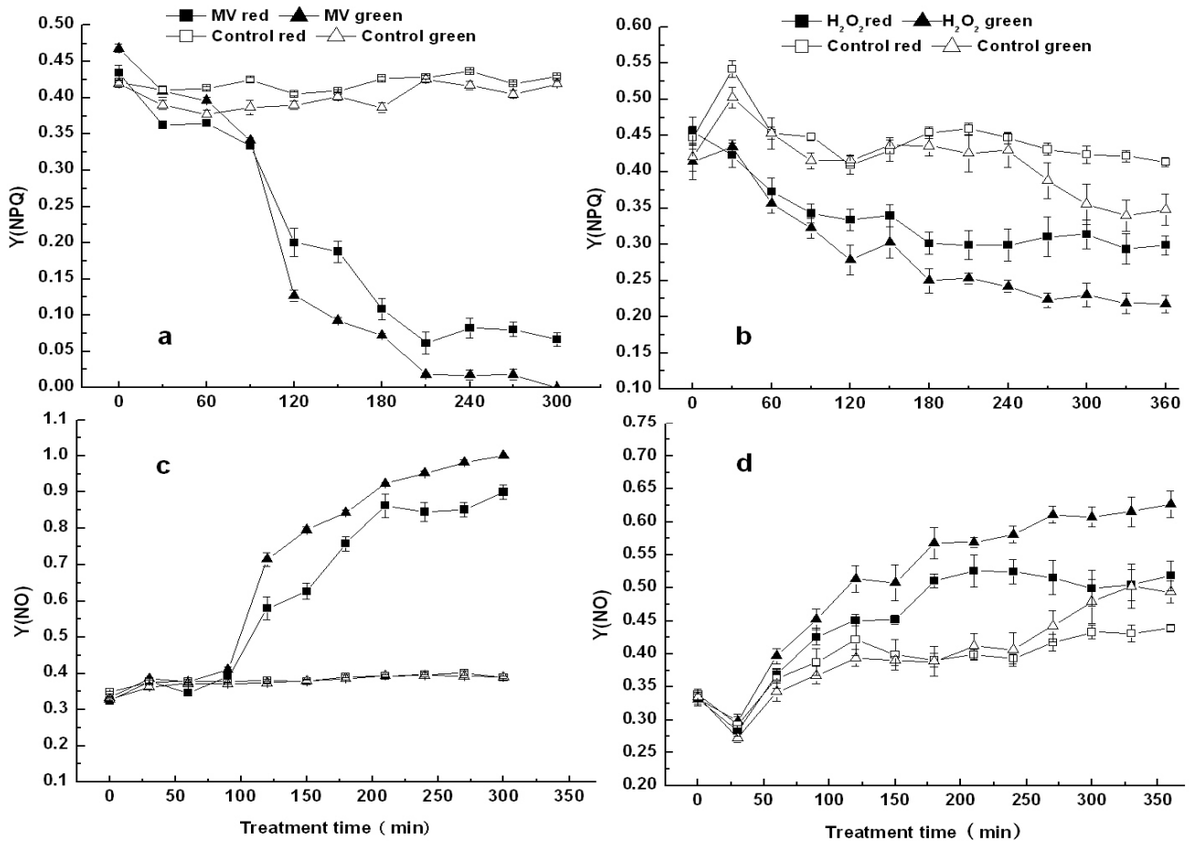

Fig.4. Effects of MV or H_2O_2 treatment on Y (NPQ) and Y (NO) in red or green leaf discs of a red cultivar of amaranth.

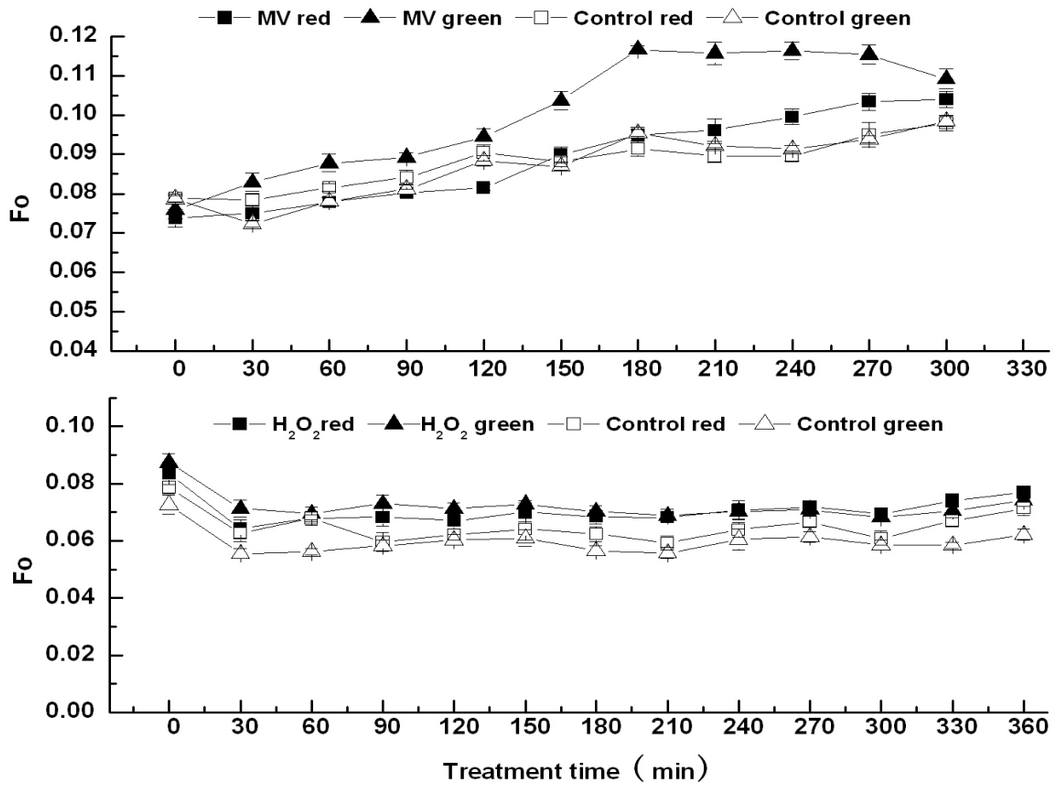

Fig. 5. Effects of MV or H_2O_2 treatment on F_o in the red or green discs from the same leaf in a red cultivar of amaranth.

Effects of photooxidation treatment on ETR: Non-cyclic photosynthetic electron transport rate (ETR) of PS II is a reflection of activity in PS II (Genty *et al.*, 1989). As shown in Fig. 6, under exogenous ROS treatment, the patterns of ETR curves of both leaf discs resembled those of Y (II) curves (Fig. 3). ETR decreased significantly under MV treatment, but was slightly greater than the control under H_2O_2 treatment. In the MV photooxidative treatment, PS II electron transfer rate increased at first (0-90 min), but declined sharply thereafter, indicating that PS II in detached leaf tissue still had a capability of dynamic regulation in responding to mild photooxidation. Subsequently, after 240 min of MV treatment, the ETR of green leaf discs reached zero, whereas the ETR of the red leaf discs remained at 5.07 ± 0.95 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Clearly, the electron transport function of PS II in red leaf discs was better protected than that in green discs under photooxidative conditions.

Content of Amaranthine in the two types of leaf discs after photooxidative treatment: Amaranthine content is the main difference of the phenotypic characteristics between the green and red leaf discs. In recent years, studies by Florian & Reinhold (2004) have pointed out that amaranthine have antioxidant properties similar to those of anthocyanin. Here in Fig. 7, under the treatment of photooxidation, amaranthine decreased in both types of leaf discs in a time-dependent manner, and amaranthine in red leaf discs declined more obviously. The decreasing rate of amaranthine was 0.0683 (Fig. 7a) and 0.0475 (Fig. 7b) by MV and H_2O_2, respectively. The result demonstrates that amaranthine, as an antioxidant in the red leaf discs, was consumed by reactions with ROS.

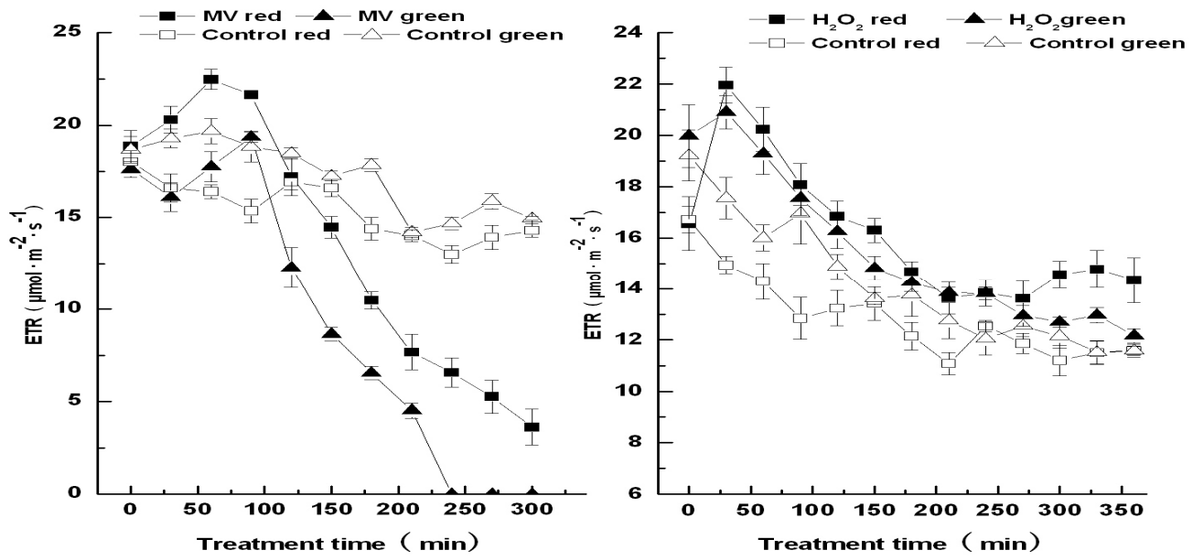

Fig. 6. Effects of MV or H_2O_2 treatment on ETR in red or green discs from the same leaf in a red cultivar of amaranth.

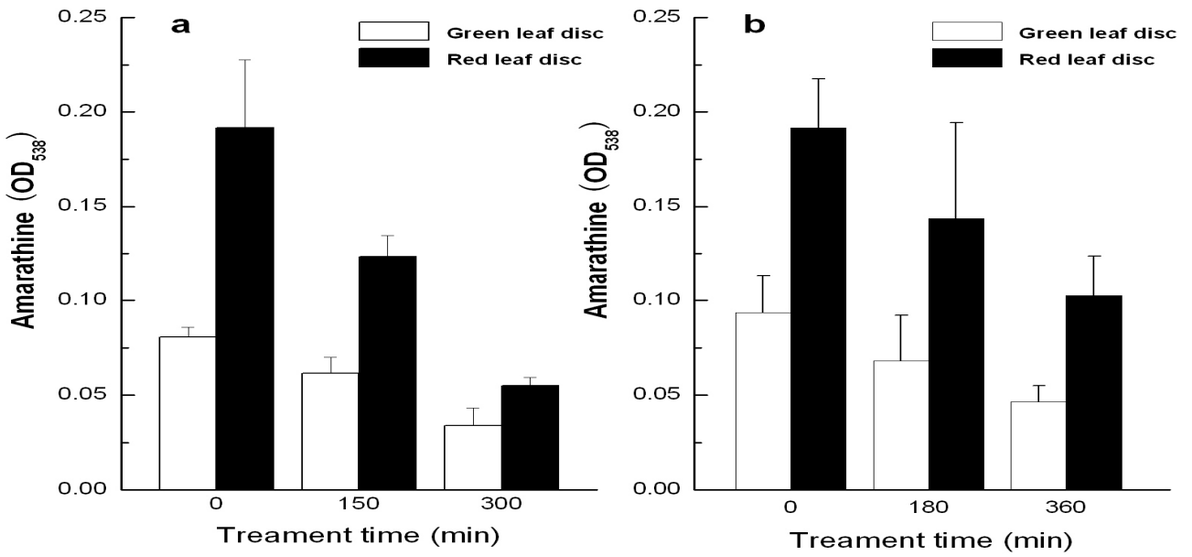

Fig. 7. Changes in the contents of amaranthine in red or green discs from the same leaf in a red cultivar of amaranth under MV (a) or H_2O_2 (b) treatment.

Discussion

As a C_4 plant, edible amaranth prefers warm weather and adapts to hot, dry or salt environments (Niu *et al.*, 2004). Among the commonly cultivated varieties, amaranth can be divided into the green amaranth, red amaranth and a red cultivar of amaranth according to the shape and color of their stems and leaves (Guan *et al.*, 1993). To ensure consistency of the material's physiological state, we selected a red cultivar of amaranth as the material and collected red and green leaf discs, differing in amaranthine content, from the same leaf.

Amaranthine is located in the vacuoles of the *Amaranthaceae* plants and is a biochrome belonging to Betacyanins of Betalain. From the test of DPPH• antioxidant activity, it demonstrated a strong capacity as an antioxidant (Cai *et al.*, 2001; Wang *et al.*, 2006). Further, Cai *et al*., (2003) reported that seven kinds of sugar beet pigments purified from *Amaranthaceae* were better antioxidants than ascorbic acid, eldrin and acacia catechuic acid. Nevertheless amaranthine possessing high ROS-scavenging activity can scavenge organelle-derived ROS (Neill & Gould, 2003). Moreover, a possibility exists particularly in stress conditions, which some ROS (H_2O_2) leaking from cellular organelles diffuses across the tonoplast entering the vacuoles (Yamasaki *et al*., 1997). Our previous study also showed that the accumulation of amaranthine and osmoregulation substances in *Amaranthus* under high temperature may be the physiological basis of its heat tolerance (Shao *et al.*, 2009). In this study, 1.93-fold higher amaranthine content in red leaf discs than in green leaf discs taken from the same leaf provided a suitable means of comparing the differential capability of antioxidation of red or green leaf discs.

At the beginning of treatment with MV or H_2O_2, the PS II activity was relatively stable, and then reduced. In the first 90 min of MV treatment, F_v/F_m did not change markedly and there was no significant enhancement in qP 、ETR and Y(II) in both types of leaf discs. F_o rose slightly. As an exogenous electron acceptor, MV can produce superoxide anions, which can induce photooxidative stress in the cell itself. This is a possible reason that PS II can maintain a relative stable activity at the beginning of the MV treatment. These results are consistent with those of exogenous $O_2^{\cdot-}$-induced chlorophyll fluorescence quenching in lettuce (Xu *et al.*, 2002). With the photooxidative treatment, F_v/F_m, Y(II) and Y(NPQ) decreased significantly, while Y(NO) and F_o increase sharply, indicating that photooxidative stress appeared in the cell, and PS II reaction center and electron transfer mediators suffered irreversible oxidative damage after a long accumulation of $O_2^{\cdot-}$. As in the MV treatment, PS II activity was nearly unaffected treated by 50 mmol·L^{-1} of H_2O_2 at the beginning of 30 min. F_v/F_m was unchanged, whereas qP, ETR and Y (II) increased, while Y (NO) and F_o decreased. With H_2O_2 treatment, Y (NO) and F_o increased, accompanied by decreases in Y (NPQ) and F_v/F_m, while Y (II), qP and ETR were still slightly higher than the control. It indicates that H_2O_2 may play the same role of Hill reagents as MV that was used in the earlier treatment (Neubauer & Schreiber, 1989). Light conversion by PS II in leaf discs was enhanced by 50 mmol·L^{-1} of H_2O_2, while excitation pressure of PS II aroused by excessive light energy was reduced. Thus, it could not affect photosynthetic electron flow. The view that the ROS have the dual-function of cellular oxidative toxicity and serving as signal molecules has been widely accepted now. Our results suggested that the relative stable state of PS II activity at the beginning of photooxidation treatment was due to induction of the cellular defense mechanisms by signal molecules of ROS.

According to the dynamic changes of chlorophyll fluorescence parameters in the green leaf discs, the sensitivity of PS II to photooxidation or the extent of oxidative damage was higher than that of the red leaf discs. After the MV and H_2O_2 treatment, the green leaf discs had higher values of initial fluorescence F_o and Y (NO) (Figs. 4 and 5) than these of the red leaf discs, but the values of Y (NPQ) (Fig. 4) and ETR (under MV treatment, Fig. 6) were lower. After 240 min MV treatment, the Y (NPQ) and qP of the green leaf discs approached zero (Fig. 5), while these two parameters of the red leaf discs still maintained a certain value. These results indicated that the PSII of red leaf discs had a higher tolerance to oxidative damage than the green leaf discs. The difference in anti-photooxidation between red discs and green discs from the same leaf is that amaranthine may be used as a detoxification mechanism to ROS in red leaf discs (Fig. 7). It seems that the strong antioxidant of red leaf discs is similar to the anti-photooxidation of purple rice leaves with abundant anthocyanin (Peng *et al.*, 2006). qp kept at relative higher level than control under H_2O_2 treatment, while MV induced an obvious decline in qp. On the other hand, there is little change in Fo under H_2O_2 treatment, whereas a significant increment in Fo under treatment of MV (Fig. 5). The results indicated that the sits of action or damage by MV and H_2O_2 may be different. Probably, MV caused severe damage in PSII reaction center, while H_2O_2 may not bring more damage to PSII.

In general, plants resistant to stresses depend on its cellular protecting mechanisms and restoration of damage capabilities. Similar to the function of anthocyanin, amaranthine, as natural anti-oxidants, also participates in the elimination of ROS to resist stresses intracellular oxidation as well as other anti-oxidants and antioxidant enzymes .Chlorophyll fluorescence quenching is a very complicated process. However, the mechanism of amaranthine underlying this process is still unclear. Previous studies reported that chloroplast ultrastructure of purple duckweed under stresses of acid rain suffered less damage than that of azolla duckweed (Lin *et al.*, 2005). MV-induced inactivation of PS II is closely associated with damage of chloroplast ultrastructure of rice (Lin *et al.*, 2009). Therefore, further studies are needed to explore whether the presence of amaranthine promotes stability of the membrane systems and chloroplast ultrastructure under photooxidation stress.

Acknowledgements

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